EXPRESS MAIL NO. EV207709506US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Donald S. Karanewsky et al.
Application No.	:	09/765,105
Filed	:	January 16, 2001
For	:	C-TERMINAL MODIFIED OXAMYL DIPEPTIDES AS INHIBITORS OF THE ICE/ced-3 FAMILY OF CYSTEINE PROTEASES

Examiner	:	David Lukton
Art Unit	:	1653
Docket No.	:	480140.442C1
Date	:	March 28, 2003

Commissioner for Patents
Washington, DC 20231

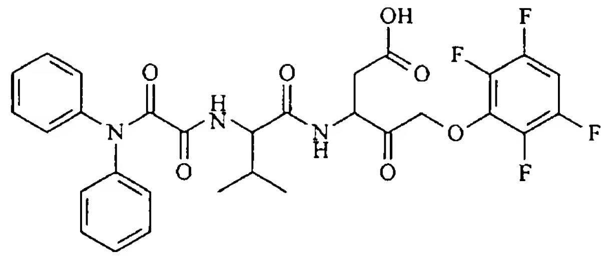
DECLARATION OF ALFRED P. SPADA, Ph.D
PURSUANT TO 37 C.F.R. §1.132

I, Alfred P. Spada, declare as follows:

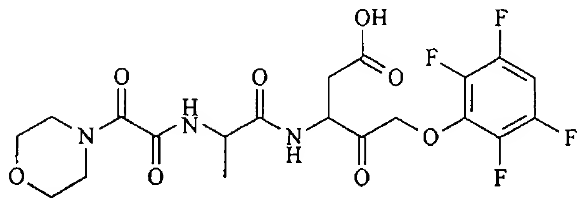
1. I am the Vice President, Pharmaceutical Development, at Idun Pharmaceuticals, the assignee of the above-identified application.

2. I have reviewed the content of this application, including the Office Action mailed December 20, 2002 and the Amendment in response thereto being filed simultaneously herewith.

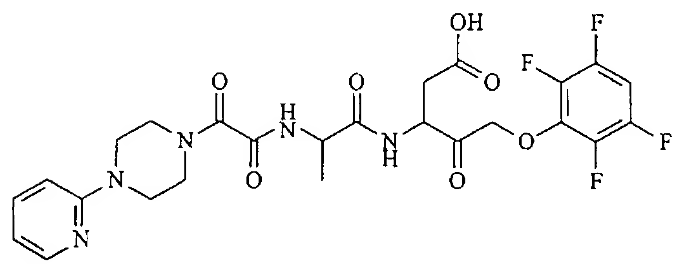
3. The following compounds (designated A through D) were made under my supervision according to the procedures disclosed in this application. In particular, Compound A was made according to Example 78, Compounds B and C were made according to Example 126, and Compound D was made according to Scheme 1 set forth on page 23.



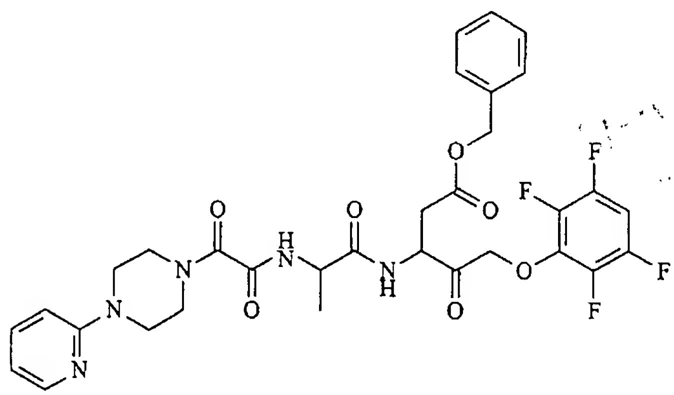
Compound A



Compound B



Compound C



Compound D

4. Compounds A through D were each assayed by the procedures set forth in the specification at page 41, lines 21 through page 42, line 15. By this assay, the IC_{50} for each of the above compounds was determined for CPP32 (*i.e.*, Caspase 3) and MCH-5 (*i.e.*, Caspase 8), the results of which are set forth in the following Table.

Table

Compound	Caspase 3 IC_{50} (μM)	Caspase 8 IC_{50} (μM)
A	0.03	0.01
B	0.03	0.47
C	0.03	0.03
D	0.05	0.03

5. The experimental results presented in the above Table demonstrate that compounds of this invention having $R^{1'}$ groups other than hydrogen also inhibit proteases *in vitro* and, more specifically, inhibit Caspase 3 and Caspase 8.

6. The following *in vivo* myocardial infarction experiment was conducted under my supervision.

7. *Surgical Preparation*: Male Wistar rats weighing approximately 200 to 300gm (Harlan, Indianapolis, IN) were acclimated in-house at least 5 days before use. Rats were anesthetized by isoflurane inhalation before an inguinalcrural incision was made, and the femoral vein was isolated. The common carotid artery was isolated through an incision in the ventral side of the neck. The distal portions of the vein and artery were ligated. A catheter filled with heparinized saline (200 U/mL) was threaded into the vessels. The vein and artery were secured by sutures. Catheters were passed subcutaneously to the dorsal nape of the neck and secured and the surgical sites secured with surgical staples. Animals were allowed to recover for at least 4 days following surgery. A femoral catheter was used for IV dosing of the test articla to animals following the myocardial ischemia/reperfusion procedure. An arterial catheter was used for arterial blood gas sampling during the surgical procedure and for perfusing the myocardium with Evan's blue dye at the time of sacrifice.

8. *Myocardial Ischemia/Reperfusion Surgical Procedures*: Rats were anesthetized by using 2% isoflurane. The chest wall was shaved and prepared. The rat was intubated and connected to a small animal volume controlled ventilator (Harvard Rodent ventilator, model 683), which pumps oxygen and isoflurane into the lungs. The ventilator rate

was 60 strokes/minute, and volume was 3.0 mL/stroke. Proper respiration was confirmed by visual observation of chest expansion and retraction during ventilated breaths and by measuring arterial pCO_2 and pO_2 before reperfusion of the left anterior descending branch (LAD). The temperature was monitored by a rectal probe, and regulated by a connected heat lamp. All surgical procedures were carried out on a heating pad and under an operating microscope. A left thoracotomy was performed. The fourth intercostals space was entered using scissors and blunt dissection. The left atrium was slightly retracted exposing the left main coronary artery system. Ligation proceeded with a 6-0 silk or monofilament suture with a tapered needle passing underneath the LAD of the left coronary artery, 1-3 mm from the tip of the normally positioned left atrium. The suture was threaded through a plastic bead and then fastened using a metal clip until proper cardiac blanching on the apex of the left ventricle was confirmed. The muscle layer of the chest was temporarily closed with 3-0 silk suture. Proper occlusion of the LAD was confirmed by changes in ST and QRS segments in the ECG taken prior to reperfusion. An arterial sample of blood was taken 3-45 minutes post occlusion to confirm appropriate ventilation of the animals. After occlusion for 60 minutes, reperfusion occurred by removal of the clip and bead, but leaving the suture in place. This allowed reperfusion of the previously ischemic bed. The chest wall was closed by using a 3-0 silk suture with one layer through the chest wall and muscle, and a second layer through the skin and subcutaneous tissue. Animals were taken off the ventilator when they began to awaken. Spontaneous respiration recovered immediately. The bolus dose was given at this time, immediately after completion of the surgical procedure, approximately 3-5 minutes post reperfusion. Buprenorphine (30 μg; 100 μL) was also injected subcutaneously at this time. The animals were fitted into the infusion harness and placed into buckets atop heating pads. They were allowed free access to food and water until they were sacrificed 24 hours after ligation. Blood samples were taken at 24 hours after ligation from the arterial catheter for determination of blood levels of test compound by LC/MS/MS.

9. *Assessment of Area at Risk and Infarct Size*: Twenty-four hours after ligation, rats were anesthetized by isoflurane inhalation. An incision was made through the abdomen and the descending abdominal aorta and the ascending vena cava was clamped off by a hemostat. The thoracic cavity was carefully entered and the left coronary artery religated with

the 6-0 suture remaining in the original position after reperfusion. To stain the viable tissue, the heart were perfused by snipping a small hole in the right atrium, and injecting 0.1% Evan's blue through the carotid catheter. The heart was excised and frozen for 1-2 hours at -20°C. The heart was then sliced transversely into 6-8 sections. To determine area at risk, each section was placed into a well in a multiple-well plate containing 2% TTC and stained for 20 minutes. The heart sections were transferred to a plate containing 10% formalin and after 5 minutes transferred to 1xPBS. The sections remained in 1xPBS at 4°C until analyzed by Digital Image Capture using ACT-1 software.

10. Using the procedures outlined in paragraphs 7-9 above, the compound of Example No. 127 of Table 7 (*see* page 75 of the application) showed a 30% reduction in infarct size when dosed at 12 mg/Kg for 24 hours.

I hereby declare that all statements made herein are, to my own knowledge, true and that all statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

Date April 15 2003

Alfred P. Spada, PhD.
Alfred P. Spada, Ph.D.

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